

Ultra Low Input mRNA-Seq

Preparing Samples for the Illumina Sequencing Platform

FOR RESEARCH USE ONLY

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Introduction

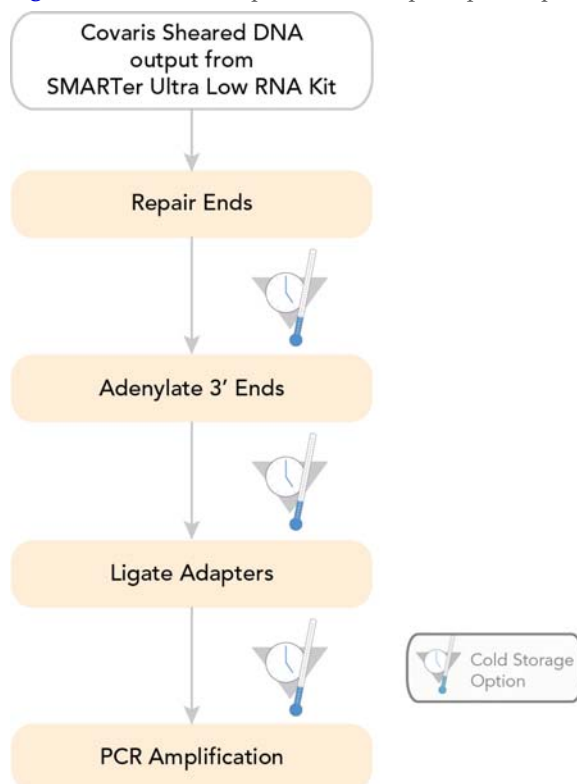
This protocol explains how to use the Illumina® Paired-End Sample Preparation Kit to convert the sheared DNA output from the Clontech SMARTer™ Ultra Low RNA Kit for Illumina Sequencing into a library of template molecules suitable for subsequent cluster generation and high-throughput DNA sequencing.

Sheared PCR amplified cDNA fragments, produced using the SMARTer Ultra Low RNA Kit, are processed through end repair procedures, the addition of a single 'A' base, and the ligation of the adapters. The products are then purified and PCR enriched to create the final cDNA library.

Sample Prep Workflow

The following figure illustrates the Ultra Low Input mRNA-Seq Sample Preparation protocol.

Figure 1 Ultra Low Input mRNA-Seq Sample Preparation Workflow



Best Practices

When preparing libraries for sequencing, you should always adhere to good molecular biology practices.

Liquid Handling

Good liquid handling measures are essential, particularly when quantifying libraries or diluting concentrated libraries for making clusters.

- Small differences in volumes ($\pm 0.5 \mu\text{l}$) can sometimes give rise to very large differences in cluster numbers ($\sim 100,000$).
- Small volume pipetting can be a source of potential error in protocols that require generation of standard curves, such as PicoGreen assays or qPCR, or those that require small but precise volumes, such as the Agilent BioAnalyzer.
- If small volumes are unavoidable, then due diligence should be taken to ensure that pipettes are correctly calibrated.
- Ensure that pipettes are not used at the volume extremes of their performance specifications.
- Care should be taken, because solutions of high molecular weight dsDNA can be viscous and not evenly dispersed, resulting in aliquot measurements that are not representative of the true concentration of the solution.

AMPure Bead Handling

The following indicates the appropriate handling methods when working with Agencourt AMPure XP Beads:

- Prior to use, allow the beads to come to room temperature.
- Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous.
- After adding the beads to the reaction, mix the solution thoroughly by pipetting up and down 10 times.
- Change the tips for each sample or when using a multichannel pipette, change the tips after each column.
- Let the mixed samples incubate for 15 minutes at room temperature for maximum recovery.
- When aspirating the cleared solution from the reaction plate and wash step, it is important to keep the plate on the magnetic stand and to not disturb the separated magnetic beads. Aspirate slowly to prevent the beads from sliding down the sides of the wells and into the pipette tips.
- For the wash steps, prepare fresh 80% ethanol. Eighty-percent ethanol tends to absorb water from the air, therefore, fresh 80% ethanol should be prepared for optimal results.
- Be sure to remove all of the ethanol from the bottom of the wells, as it may contain residual contaminants.
- Remove the reaction plate from the magnetic stand and let it air-dry at room temperature. Allow for the complete evaporation of residual ethanol, as it impacts the performance of the subsequent reactions. Illumina recommends at least 15 minutes drying time, but a longer drying time may be required.
- Use the QIAGEN EB for DNA elution. gently pipette up and down 10 times making sure the liquid comes in contact with the beads and that the beads are resuspended homogeneously.

DNA Input Recommendations

Input DNA Library Quality

High quality SMARTer Ultra Low RNA amplified material is essential for the success of the Ultra Low Input mRNA-Seq Sample Prep. Verify the size distribution of the SMARTer Ultra Low RNA amplified material by running 1 μ l of the PCR product (of 12 μ l purified with AMPure XP Beads) on a Agilent Technologies 2100 Bioanalyzer using a Agilent High Sensitivity DNA chip. Contaminated products should be discarded.



NOTE

Reference the Clontech *SMARTer Ultra Low RNA Kit User Manual*.

Figure 2 Agilent Profile - Clean Sample

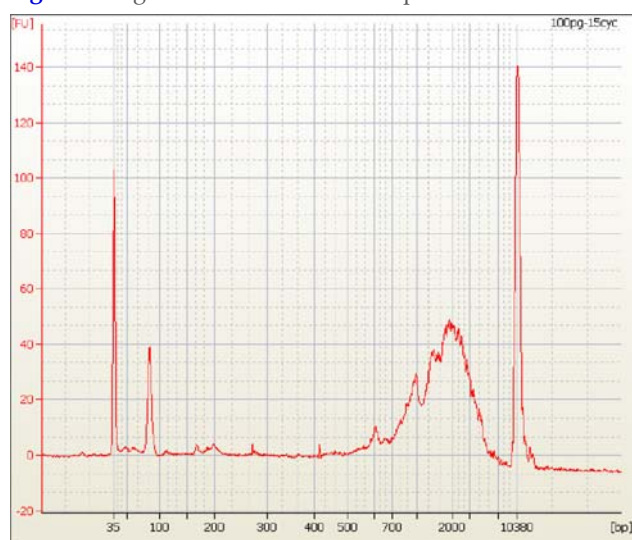


Figure 3 Agilent Profile - Contaminated Sample

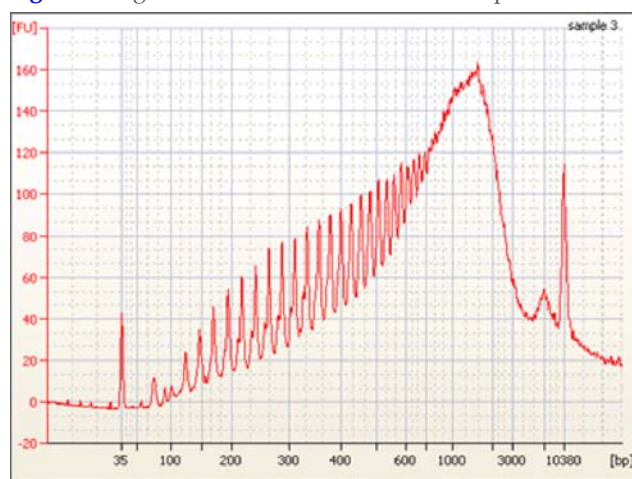
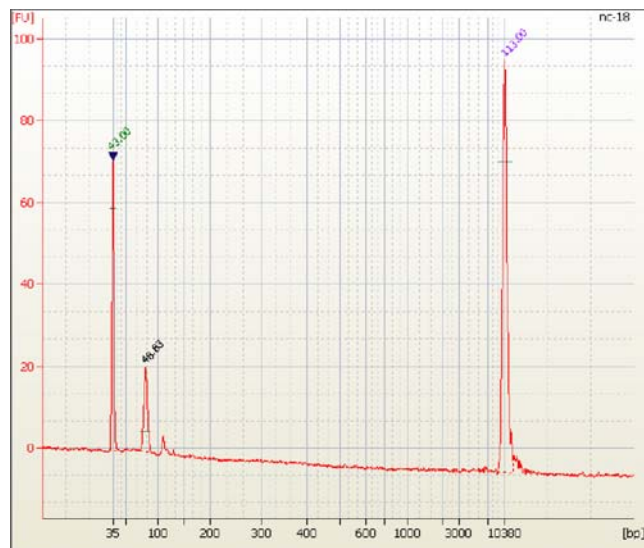


Figure 4 Agilent Profile - Negative Control

Input DNA Library Quantification

Illumina recommends a minimum of 2 ng DNA per library for the Ultra Low Input mRNA-Seq Sample Prep protocol. The ultimate success of the library preparation strongly depends on using an accurately quantified amount of input DNA. Therefore, correct quantification of the DNA library is essential.

Quantify the concentration of the DNA library purified with AMPure XP Beads by manually integrating a peak between 400–9000 bp of the Bioanalyzer trace obtained from *Input DNA Library Quality* and multiplying the concentration by 12 μ l to determine the total amount of library.

Consumables and Equipment

Check to ensure that you have all of the necessary user-supplied consumables and equipment before proceeding to sample preparation.

Table 1 User-Supplied Consumables

Consumable	Supplier
0.2 ml nuclease-free thin-wall PCR tubes	USA Scientific, part # 1402-4700
1.5 ml nuclease-free, non-sticky microcentrifuge tubes	USA Scientific, part # 1415-2600
96-well V-bottom plates (500 µl)	VWR, catalog # 47743-996
Agencourt AMPure XP PCR Purification Kit (60 ml)	Beckman Coulter Genomics, part # 63881
Covaris sheared DNA (75 µl)	User supplied output from SMARTer Ultra Low RNA Kit, Clontech part # 634935
Freshly Prepared 80% Ethanol	General lab supplier
MicroAmp Clean Adhesive Film	Applied Biosystems, part # 4306311
Nuclease-free water	General lab supplier
Paired-End Sample Prep Kit	Illumina, catalog # PE-102-1001 (10 samples) or PE-102-1002 (40 samples)
QIAGEN EB Buffer	QIAGEN, part # 19086

Table 2 User-Supplied Equipment

Equipment	Supplier
Magnetic stand-96	Ambion, part # AM10027
Thermal cycler	General lab supplier

SMARTer Ultra Low RNA Protocol

Prior to Ultra Low Input mRNA-Seq Sample Preparation, you must first PCR amplify and fragment the cDNA library using the SMARTer Ultra Low RNA Kit (Reference the Clontech *SMARTer Ultra Low RNA Kit User Manual*). To ensure high-quality DNA input for this protocol, see *DNA Input Recommendations* on page 5.

Perform End Repair

DNA fragmentation by physical methods produces heterogeneous ends, comprising a mixture of 3' overhangs, 5' overhangs, and blunt ends. The overhangs will be of varying lengths and ends may or may not be phosphorylated. This step converts the overhangs resulting from fragmentation into blunt ends using the T4 DNA Polymerase and Klenow Enzyme. The 3' to 5' exonuclease activity of these enzymes removes 3' overhangs and the polymerase activity fills in the 5' overhangs. In addition, T4 PNK in this reaction phosphorylates the 5' ends of the DNA fragments.

User-Supplied Consumables

- 0.2 ml nuclease-free thin-wall PCR tubes (2)
- 1.5 ml nuclease-free, non-sticky microcentrifuge tube
- 96-well V-bottom plate (500 µl)
- AMPure XP Beads
- Covaris Sheared DNA (output from SMARTer Ultra Low RNA Kit)
- Freshly Prepared 80% Ethanol (EtOH)
- MicroAmp clean adhesive film
- Paired-End Sample Prep Kit content:
 - 10 mM dNTP Mix
 - Klenow Enzyme
 - T4 DNA Ligase Buffer with 10 mM ATP
 - T4 DNA Polymerase
 - T4 PNK
- QIAGEN EB

Procedure

- 1 Preheat a thermal cycler to 20°C.
- 2 Prepare the following reaction mix in a new 0.2 ml nuclease-free thin-wall PCR tube. Multiply each volume by the number of samples being prepared. Prepare 10% extra reagent mix if you are preparing multiple samples.

Reagent	Volume (µl)
Covaris Sheared DNA (2–10 ng)	75
T4 DNA Ligase Buffer with 10 mM ATP	10
10 mM dNTP Mix	4
T4 DNA Polymerase	5
Klenow Enzyme	1
T4 PNK	5
Total Volume per Sample	100

- 3 Incubate the sample on a thermal cycler for 30 minutes at 20°C.
- 4 Transfer 100 µl of the sample to each well of a new 96-well V-bottom plate.

- 5 Vortex the AMPure XP Beads until they are well dispersed, then add 180 μ l of the mixed AMPure XP Beads to each well of the 96-well V-bottom plate containing the reaction mix. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 6 Incubate the plate at room temperature for 8 minutes.
- 7 Place the plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.
- 8 Remove and discard all of the supernatant from each well of the plate. Take care not to disturb the beads. Change the tip after each sample.

**NOTE**

Leave the plate on the magnetic stand while performing the following 80% EtOH wash steps (9–11).

- 9 With the plate remaining on the magnetic stand, add 180 μ l of freshly prepared 80% EtOH to each well of the plate without disturbing the beads.
- 10 Incubate the plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well. Take care not to disturb the beads. Change the tip after each sample.
- 11 Repeat steps 9 and 10 once for a total of two 80% EtOH washes.
- 12 Remove the plate from the magnetic stand and let the plate stand at room temperature for 15 minutes to dry.
- 13 Resuspend the dried pellet in each well with 32 μ l QIAGEN EB. Gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.
- 14 Incubate the plate at room temperature for 2 minutes.
- 15 Place the plate on the magnetic stand at room temperature for 2 minutes or until the liquid appears clear.
- 16 Transfer 32 μ l of the clear supernatant from each well of the plate to a new 0.2 ml nuclease-free thin-wall PCR tube. Change the tip after each sample.

**SAFE STOPPING POINT**

If you do not plan to proceed to *Adenylate 3' Ends* immediately the protocol can be safely stopped here. If you are stopping, transfer the sample to a 1.5 ml nuclease-free, non-sticky microcentrifuge tube and store it at -15° to -25°C for one day.

Adenylate 3' Ends

A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation.

User-Supplied Consumables

- 0.2 ml Nuclease-free thin-wall PCR tubes (2)
- 1.5 ml nuclease-free, non-sticky microcentrifuge tube
- 96-well V-bottom plate (500 µl)
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- MicroAmp clean adhesive film
- Paired-End Sample Prep Kit content:
 - 1 mM dATP
 - Klenow Buffer
 - Klenow exo -
- QIAGEN EB

Procedure

- 1 Preheat a thermal cycler to 37°C.
- 2 Prepare the following reaction mix in a new 0.2 ml nuclease-free thin-wall PCR tube. Multiply each volume by the number of samples being prepared. Prepare 10% extra reagent mix if you are preparing multiple samples.

Reagent	Volume (µl)
Eluted DNA	32
Klenow Buffer	5
1 mM dATP	10
Klenow exo -	3
Total Volume per Sample	50

- 3 Incubate the sample on a thermal cycler for 30 minutes at 37°C.
- 4 Transfer 50 µl of the sample to each well of a new 96-well V-bottom plate.
- 5 Vortex the AMPure XP Beads until they are well dispersed, then add 90 µl of the mixed AMPure XP Beads to each well of the 96-well V-bottom plate containing the reaction mix. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 6 Incubate the plate at room temperature for 8 minutes.
- 7 Place the plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.
- 8 Remove and discard all of the supernatant from each well of the plate. Take care not to disturb the beads. Change the tip after each sample.

**NOTE**

Leave the plate on the magnetic stand while performing the following 80% EtOH wash steps (9–11).

- 9 With the plate remaining on the magnetic stand, add 180 μ l of freshly prepared 80% EtOH to each well of the plate without disturbing the beads.
- 10 Incubate the plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well. Take care not to disturb the beads. Change the tip after each sample.
- 11 Repeat steps 9 and 10 once for a total of two 80% EtOH washes.
- 12 Remove the plate from the magnetic stand and let the plate stand at room temperature for 15 minutes to dry.
- 13 Resuspend the dried pellet in each well with 19 μ l QIAGEN EB. Gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.
- 14 Incubate the plate at room temperature for 2 minutes.
- 15 Place the plate on the magnetic stand at room temperature for 2 minutes or until the liquid appears clear.
- 16 Transfer 19 μ l of the clear supernatant from each well of the plate to a new 0.2 ml nuclease-free thin-wall PCR tube. Change the tip after each sample.

**SAFE STOPPING POINT**

If you do not plan to proceed to *Ligate Adapters* immediately the protocol can be safely stopped here. If you are stopping, transfer the sample to a 1.5 ml nuclease-free, non-sticky microcentrifuge tube and store it at -15° to -25°C for one day.

Ligate Adapters

This process ligates adapters to the ends of the DNA fragments. The reaction adds distinct sequences to the 5' and 3' ends of each strand in the genomic fragment. Later in the workflow, additional sequences are added by tailed primers during PCR. These additional sequences are necessary for library amplification on the flow cell during cluster formation.

User-Supplied Consumables

- 0.2 ml Nuclease-free thin-wall PCR tubes (3)
- 96-well V-bottom plate (500 µl)
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- MicroAmp clean adhesive film
- Paired-End Sample Prep Kit content:
 - DNA Ligase Buffer 2X
 - Ultra-pure water
 - PE Adapter Oligo Mix
 - T4 DNA Ligase
- QIAGEN EB

Procedure

- 1 Prepare a dilution of the paired-end adapter in ultra-pure water in a new 0.2 ml nuclease-free thin-wall PCR tube:

Covaris Input DNA (ng)	Adapter:Water Dilution
8–50	1:9
4–8	1:14
<1–4	1:19

- 2 Prepare the following reaction mix in a new 0.2 ml nuclease-free thin-wall PCR tube. Multiply each volume by the number of samples being prepared. Prepare 10% extra reagent mix if you are preparing multiple samples.

Reagent	Volume (µl)
Eluted DNA	19
DNA Ligase Buffer 2X	25
PE Adapter Oligo Mix (diluted)	1
T4 DNA Ligase	5
Total Volume per Sample	50

- 3 Incubate the sample at room temperature for 15 minutes.
- 4 Transfer 50 µl of the sample to each well of a new 96-well V-bottom plate.

- 5 Vortex the AMPure XP Beads until they are well dispersed, then add 80 μ l of the mixed AMPure XP Beads to each well of the 96-well V-bottom plate containing the reaction mix. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 6 Incubate the plate at room temperature for 8 minutes.
- 7 Place the plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.
- 8 Remove and discard all of the supernatant from each well of the plate. Take care not to disturb the beads. Change the tip after each sample.

**NOTE**

Leave the plate on the magnetic stand while performing the following 80% EtOH wash steps (9–11).

- 9 With the plate remaining on the magnetic stand, add 180 μ l of freshly prepared 80% EtOH to each well of the plate without disturbing the beads.
- 10 Incubate the plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well. Take care not to disturb the beads. Change the tip after each sample.
- 11 Repeat steps 9 and 10 once for a total of two 80% EtOH washes.
- 12 Remove the plate from the magnetic stand and let the plate stand at room temperature for 15 minutes to dry.
- 13 Resuspend the dried pellet in each well with 23 μ l QIAGEN EB. Gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.
- 14 Incubate the plate at room temperature for 2 minutes.
- 15 Place the plate on the magnetic stand at room temperature for 2 minutes or until the liquid appears clear.
- 16 Transfer 23 μ l of the clear supernatant from each well of the plate to a new 0.2 ml nuclease-free thin-wall PCR tube. Change the tip after each sample.

**SAFE STOPPING POINT**

If you do not plan to proceed to *Enrich DNA Fragments* immediately the protocol can be safely stopped here. If you are stopping, transfer the sample to a 1.5 ml nuclease-free, non-sticky microcentrifuge tube and store it at -15° to -25°C for one day.

Enrich DNA Fragments

This process uses PCR to selectively enrich those DNA fragments that have adapter molecules on both ends and to amplify the amount of DNA in the library for accurate quantification. PCR is performed with two primers that anneal to the ends of the adapters. The number of PCR cycles should be minimized to avoid skewing the representation of the library.

The PCR amplification process performs four key functions:

Add Sequences – Additional sequences are added to the ends of the adapters so that the final amplified templates contain sequences to enable hybridization with primers bound to the flow cell surface for cluster generation.

Enrich Fragments – PCR enriches fragments that have adapters ligated on both ends. Fragments with only one or no adapters on their ends are by-products of inefficiencies in the ligation reaction. Neither species can be used to make clusters, as fragments without any adapters cannot hybridize to surface-bound primers in the flow cell, and fragments with an adapter on only one end can hybridize to surface bound primers, but cannot form clusters. However, the presence of these incomplete ligation products can lead to an overestimation of the final quantity of library DNA if spectrophotometric or fluorometric quantification is performed. In contrast, these ligation products are not quantified by qPCR.

Enrich Templates – PCR enriches templates that include the non-template 'A' nucleotide added during the A-tailing process and therefore eliminates adapter dimers. This is accomplished by using proprietary, modified primers that are completely resistant to the 3'–5' exonuclease activity of the Phusion (Finnzymes Oy) polymerase used for PCR. These primers reach all the way to the non-templated 'A' before the start of the genomic sequence. If the 'A' is not present (as in the case for an adapter dimer), then the terminal 'T' on the primer will mismatch and not extend.

Provides Material – PCR provides enough material to enable reliable quantification of the final library if spectrophotometric or fluorometric methods are used.

User-Supplied Consumables

- 0.2 ml nuclease-free thin-wall PCR tube
- 1.5 ml nuclease-free, non-sticky microcentrifuge tube
- 96-well V-bottom plate (500 µl)
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- MicroAmp clean adhesive film
- Paired-End Sample Prep Kit content:
 - Phusion™ DNA Polymerase (Finnzymes Oy)
 - PCR Primer PE 1.0
 - PCR Primer PE 2.0

Procedure

- 1 Prepare the following reaction mix in a new 0.2 ml nuclease-free thin-wall PCR tube on ice. Multiply each volume by the number of samples being prepared. Prepare 10% extra reagent mix if you are preparing multiple samples.

Reagent	Volume (μl)
DNA	23
Phusion DNA Polymerase	25
PCR Primer PE 1.0	1
PCR Primer PE 2.0	1
Total Volume per Sample	50

- 2 Amplify the PCR tube in the thermal cycler, with the lid closed:
 - a 98°C for 30 seconds
 - b 15 cycles of:
 - 98°C for 10 seconds
 - 60°C for 30 seconds
 - 72°C for 30 seconds
 - c 72°C for 5 minutes
 - d Hold at 4°C
- 3 Transfer 50 μl of the sample to each well of a new 96-well V-bottom plate.
- 4 Vortex the AMPure XP Beads until they are well dispersed, then add 80 μl of the mixed AMPure XP Beads to each well of the 96-well V-bottom plate containing 50 μl of the PCR amplified library. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 5 Incubate the plate at room temperature for 8 minutes.
- 6 Place the plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.
- 7 Remove and discard all of the supernatant from each well of the plate. Take care not to disturb the beads. Change the tip after each sample.



NOTE

Leave the plate on the magnetic stand while performing the following 80% EtOH wash steps (8–10).

- 8 With the plate remaining on the magnetic stand, add 180 μl of freshly prepared 80% EtOH to each well of the plate without disturbing the beads.
- 9 Incubate the plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well. Take care not to disturb the beads. Change the tip after each sample.
- 10 Repeat steps 8 and 9 once for a total of two 80% EtOH washes.
- 11 Remove the plate from the magnetic stand and let the plate stand at room temperature for 15 minutes to dry.

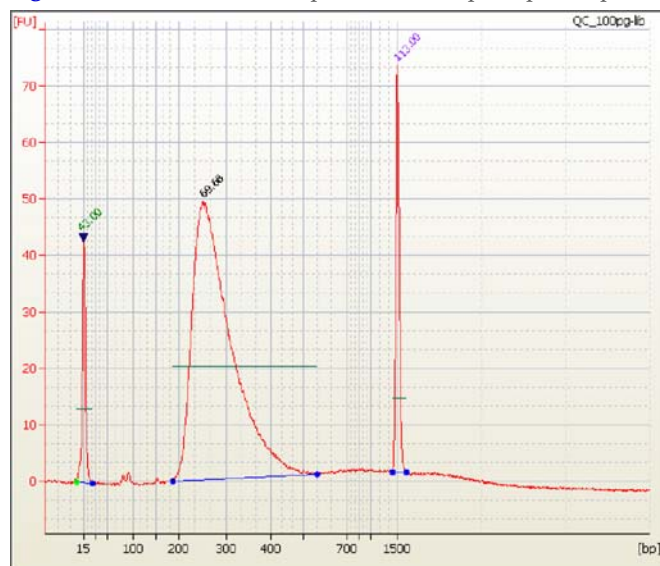
- 12** Resuspend the dried pellet in each well with 15 μ l QIAGEN EB. Gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.
- 13** Incubate the plate at room temperature for 2 minutes.
- 14** Place the plate on the magnetic stand at room temperature for 2 minutes or until the liquid appears clear.
- 15** Transfer 15 μ l of the clear supernatant from each well of the plate to a new 1.5 ml nuclease-free, non-sticky microcentrifuge tube and store it at -20°C.

Validate Library

Illumina recommends performing the following quality control analysis on your sample library to quantify the DNA concentration.

- 1 Load 1 μ l of the resuspended construct on an Agilent Technologies 2100 Bioanalyzer using a DNA specific chip such as the Agilent DNA-1000.

Figure 5 Final Ultra Low Input mRNA-Seq Sample Prep Library Bioanalyzer Trace



- 2 Check the size, purity, and concentration of the sample. The final product should be a distinct band at approximately 250 bp.